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Abstract

The therapeutic strategies proposed currently for bone sarcomas are based on neo-adjuvant chemotherapy, delayed en-bloc wide resection, and adjuvant chemotherapy. Unfortunately, bone sarcomas are characterized by high rates of poor drug response, with a high risk of drug resistance, local recurrence and/or a high propensity for induced metastases. The pathogenesis of bone sarcomas is strongly associated with dysregulation of local bone remodeling and increased osteolysis that plays a part in tumor development. In this context, bisphosphonates (BPs) have been proposed as a single agent or in combination with conventional drugs to block bone resorption and the vicious cycle established between bone and sarcoma cells. Pre-clinical *in vitro* studies revealed the potential "anti-tumor" activities of nitrogen-bisphosphonates (N-BPs). In pre-clinical models, N-BPs reduced significantly primary tumor growth in osteosarcoma and Ewing sarcoma, and the installation of lung metastases. In chondrosarcoma, N-BPs reduced the recurrence of local tumors after intralesional curettage, and increased overall survival. In pediatric and adult osteosarcoma patients, N-BPs have been assessed in combination with conventional chemotherapy and surgery in randomized phase 3 studies with no improvement in clinical outcome. The lack of benefit may potentially be explained by the biological impact of N-BPs on macrophage differentiation/recruitment which may alter $CD8^+$-T lymphocyte infiltration. Thanks to their considerable affinity for the mineralized extracellular matrix, BPs are an excellent platform for drug delivery in malignant bone sites with reduced systemic toxicity, which opens up new opportunities for their future use.

1. Introduction

Bone sarcomas are malignant primary tumors in bone, represented by heterogeneous neoplastic entities of mesenchymal origin [1]. Based on their low frequency, with less than 2% of malignant tumors registered in the EUROCARE database, bone sarcomas are considered to be orphan tumors. Osteosarcoma, Ewing sarcoma and chondrosarcoma are the main members of this tumor family. Osteosarcoma and Ewing sarcomas are associated with a high ability to establish metastatic nodules in the lungs and bone, and chondrosarcomas are characterized by a high risk of local recurrence and metastatic disease for high-grade histological subtypes [1].

Initial development of bone sarcomas is explained by the conjunction of oncogenic events (e.g. mutation of *p53*, *Rb*, *EXT; fusion gene: EWS-FLi*) and dysregulation of the microenvironment which markedly contributes to the pathogenesis of the disease [3-7]. As bone is the natural initial environment for bone sarcomas, bone cells cooperate with tumor cells and facilitate their proliferation and migration. Osteoblasts and mesenchymal stem cells communicate directly with bone sarcoma cells using GAP junctions [8,9], but also indirectly through the release of cytokines/growth factors and the production of extracellular vesicles [10]. All bone sarcoma entities are characterized by a major alteration to local bone remodeling that can lead to bone fractures. This bone degradation is linked to the recruitment of osteoclast precursors and/or the activation of mature osteoclasts by soluble factors secreted by cancer cells. M-CSF and RANKL are the two main protagonists related to this process [3]. In turn, activated osteoclasts degrade bone and induce the release of growth factors initially trapped in the bone extracellular matrix. TGF-β is one of the factors that stimulates cancer cell proliferation and cell migration [11,12]. A vicious cycle between resident cells and tumor cells is then established and plays a part in bone sarcoma growth and the associated metastatic process (Figure 1).

The activation of osteoclastogenesis in bone sarcomas has stimulated therapeutic interest in osteoclast targeting agents as a mean of blocking the functional dependency of tumor growth related to bone resorption [13,14]. Bisphosphonates (BPs) have been used successfully for skeletal diseases associated with exacerbated activation of osteoclasts. In this context, BPs were initially used in Paget's disease and malignant hypercalcemia such as bone metastases, and their clinical indications have progressively been extended to osteoporosis [15]. Osteoclasts/osteoclast precursors were the first targets of BPs, but decades of studies have

clearly demonstrated that BPs have numerous cellular targets including macrophages, endothelial cells and immune cells, and could also modulate the biology of cancer cells, at least *in vitro*. BP use has been studied extensively in prostate and breast cancer patients with bone metastases, and has been also assessed in bone sarcomas. The aim of the present review is to give a brief overview of the mechanisms of action of BPs in bone sarcomas, and to discuss their clinical relevance in these tumor entities.

1. BPs and osteosarcoma

Osteosarcoma is the main bone sarcoma and represents around 50% of malignant primary bone tumors [1]. Osteosarcomas are characterized by a proliferation of mesenchymal cells responsible for the formation of an osteoid matrix. Even though a small number of osteosarcomas can be directly related to predisposition factors such as a mutation of *p53* (Li Fraumeny syndrome), fibrous dysplasia, Paget's disease and irradiation, most osteosarcomas are of unknown cause. The former entities are named secondary osteosarcomas and are more frequently observed around the age of 60 years, and the latter are called primary or idiopathic osteosarcomas and are characterized by a peak of incidence around the age of 18 years. They can be classified according their localization (e.g. medullary, intracortical, and juxtacortical osteosarcomas), as well as according to their histological subtypes (e.g. fibroblastic, chondroblastic, telangiectatic). Initial osteosarcoma growth is associated with a phase of osteoclastic activation/recruitment, which has led to the use of BPs [16]. Osteosarcoma can be then considered as a bone-forming tumor functionally related to osteoclasts. The local microenvironment is also characterized by a marked immune infiltrate composed of a high level of macrophages and lymphocytes [17].

1. BPs modulate the local osteosarcoma microenvironment

1.1. BP targeting of osteoclasts in osteosarcoma

Osteoclasts and their precursors are the main cellular targets of BPs in bone, and inhibit bone resorption by inducing osteoclast cell death [18]. Interestingly, it has been observed that administration of nitrogen-containing BPs (N-BPs) such as zoledronate may interfere with the mechanical properties of bone, by altering bone remodeling and inhibiting the biological functions of both osteoclasts and osteoblasts [19]. For example, N-BP-treated mice showed reduced bone growth [19]. Low doses of N-BPs (0.02-0.025 mg/kg) were associated with a frequency of hypocalcemia that was significantly reduced by decreasing the dose of N-BPs used (0.0125 mg/kg). However, these doses were lower than those used in an oncological

context [20]. Higher doses of N-BPs (50 µg/kg every 2 days × 10) were then assessed in growing mice to determine their potential harmful impact on bone [21]. N-BPs had a transient inhibitory effect on bone length with bone-growth arrest during treatment. These effects were associated to a strong inhibition of resorption leading to an increase of the metaphyseal bone density [22, 23]. Osteoblast and osteoclast biomarkers decreased in serum. The bone growth arrest was stopped at the end of N-BP treatment. A young patient with an osteosarcoma treated with conventional chemotherapy combined with N-BP (50 µg/kg every 2 days × 10 iv injections) exhibited an increase in bone mineral density at the growth plate related to inhibition of bone resorption that was transient, as shown by X-ray radiography [21]. N-BPs inhibit osteoclast function transiently and could be used in young patients. Zoledronate can be safely combined with conventional chemotherapy with maximal tolerated doses mg/m^2 (max 4 mg) for patients with metastatic osteosarcoma [24]. However, more recently, Lezot *et al.* showed that high doses of zoledronate irreversibly disturbed teeth eruption and elongation, and delayed skull bone formation in a preclinical mouse model [25].

BPs were then assessed in immunocompetent and immunodeficient murine models of osteosarcoma. In an immunocompetent rat model (OSRGa cells), zoledronate reduced the growth of primary tumors and inhibited the osteolytic lesions associated with tumor growth [26]. When combined with conventional chemotherapy (Ifosfamide), it was more effective than single agents for preventing tumor recurrence and improving tissue repair. In immunodeficient mice (inoculation of human Saos2 osteosarcoma cells), zoledronate (s.c. twice weekly at 120 microg/kg) inhibited tumor growth in the orthotopic site [27]. On the contrary, Labrinidis *et al.* did not observe any effect of zoledronate on tumor growth when given as either a weekly or a single dose of 100 microg/kg (inoculation of human K-Hos osteosarcoma cells) [28]. However, they did observe a beneficial effect of zoledronate in that it prevented osteolytic lesions and significantly limited the amount of malignant ectopic bone formation [28]. In a xenogenic model (athymic rats) of canine osteosarcoma, zoledronate improved bone architecture and its mechanical properties when combined with PTH [29]. Similarly, Wolfe *et al*. observed that zoledronate had a significant bone protector effect in a murine model of canine osteosarcoma (OSCA40 cells inoculated intratibially in nude mice followed by limb amputation), with no effective inhibition of lung metastases [30]. In two different models (immunocompetent: murine POS-1 cells inoculated in C3H/He mice [31]; immunodeficient: murine LM8 cells injected into nude mice [32]), high doses of zoledronate significantly reduced the establishment of lung metastases and improved overall survival.

These results were controversial, and zoledronate did not show any therapeutic benefit in immunodeficient mice (human K-HOS cells) [28] nor in an immunocompetent rat osteosarcoma model (MSK- 8G osteosarcoma cells) [33]. The literature nevertheless described sporadic but encouraging progression-free survival in patients with high-grade osteosarcoma and progressive metastatic disease after polychemotherapy and treated with zoledronate alone [34]. However, two independent phase III clinical trials did not demonstrate any therapeutic benefit of combining zoledronate with conventional chemotherapy in pediatric and adult metastatic osteosarcoma patients [35,36]. These discrepancies may be explained by the differential origin of the cell lines used, the cancer cell inoculation protocol (e.g. intratibial, paraosseous sites), or the strain of mice/rat models (C3H/HE, Balbc nu/nu, NMRI Nude, Sprague Dawley, Fisher 344). Furthermore, there are major conflicts in the literature regarding the involvement of osteoclasts in metastasis. There is some evidence that osteoclasts are directly associated with poor outcomes in osteosarcoma [37] that may be related to the pro-angiogenic role of osteoclasts [38]. In contrast, other studies show that osteoclasts localized at the site of primary tumors are capable of preventing the metastatic process [37]. This apparent dual activity of osteoclasts may be related to the stage of the disease and should be reconsidered in light of recent knowledge acquired about osteoclasts [39]. Osteoclasts belong to a very heterogeneous family composed of multinucleated cells with pro-inflammatory and immune-tolerant activities that may explain the differential therapeutic benefit of BPs depending on the subsets of osteoclast.

1.2. Targeting of immune cells by BPs

Tumor-associated macrophages (TAMs) are recruited by cancer cells and infiltrate the tumor mass. Their polarization appears to be controlled by the local microenvironment [40]. In 2015, Junankar *et al*. definitively established that macrophages are the main targets of BPs and consequently that the antitumor activity observed in bone and outside the skeleton is mediated at least partly via TAMs [41]. Osteosarcoma tissues are markedly infiltrated by TAMS [17] and their polarization is crucial in tumor development and the initiation of the metastatic process [42,43]. Zoledronate prevents M2-macrophage polarization by targeting the mevalonate pathway and RhoA geranyl-geranylation [44]. In addition, zoledronate promotes M1-macrophage polarization though the TL4 pathway [45]. The dysregulation of M1/M2 macrophage polarization induced by N-BPs may alter CD8+-T lymphocyte infiltration [46]. This functional impact on macrophage polarization may explain the lack of therapeutic benefit of N-BPs in osteosarcoma patients [44,45].

N-BPs induce intracellular accumulation of isopentenyl diphosphate (IPP) and dimethylallyl diphosphate (DMAPP) by inhibiting farnesyl pyrophosphate synthase [47-49]. Treatment of cancer cells *in vitro* by zoledronate results in the accumulation of IPP/DMAPP, cell death and then the release of the phosphoantigen that can trigger activation of Vγ9Vδ□T lymphocytes [47-48]. Similarly, the peripheral blood monocytes targeted by N-BPs are responsible for Vγ9Vδ□T lymphocyte activation, induced through accumulation of phosphoantigens and in a cell contact-dependent manner [49]. Vγ9Vδ□T lymphocyte activation by N-BPs was a promising therapeutic line in osteosarcoma [50-54]. However, Fleming *et al*. recently revealed that γδT cells may promote tumor progression by mimicking Treg/Th2-like cells and inducing local immunosuppression (e.g. modulation of dendritic cell activity and inhibition of PD1-PDL1 axis) [55].

1.3. Direct *in vitro* activity of BPs in osteosarcoma

Numerous publications have demonstrated the direct effects of BPs on osteosarcoma cells *in vitro* [56-68]. N-BPs inhibited the proliferation of osteosarcoma cells and induced cell death by blocking cell cycle progression in the S, G2/M phases independently of their p53 and Rb status [66,67]. This cell cycle arrest was due to activation of the intra-S DNA damage checkpoint, and more particularly to an increase in ATR, P-chk1, Wee1, and P-cdc2 phophorylation levels and a decrease in cdc25c [66]. Zoledronate induced Rb phosphorylation and stimulated the progression in the G1/S phase of Rb wild-type osteosarcoma cells, leading to their accumulation in the S phase, where they became more sensitive to DNA damage. Failing to repair their DNA damage, the osteosarcoma cells initiated a feedback process by inhibiting Rb phosphorylation through cyclin/cdk complex regulation [66]. In addition, zoledronate induced atypical apoptosis [66,68]. Zoledronate-induced cell death was independent of caspase activation and involved the mitochondria pathway. N-BP-associated cell death was characterized by nuclear alterations and dysregulation of the Bax/Bcl2 balance. It also implicated up-regulation of mitochondrial permeability, with translocation of apoptosis-inducing factor (AIF) and endonuclease-G (EndoG) [66,68]. At the end of the molecular process, N-BPs markedly altered cytoskeletal organization and cell junctions, leading to inhibited cell migration and disturbance of focal adhesion kinases. More recently, AKT/GSK-3β/β-Catenin signaling was also associated with N-BP-induced cell death [56]. Both N-BPs and non-N-BPs also directly modulated osteosarcoma cell migration and

invasion through the inhibition of metalloproteinase expression [69-71]. Finally, N-BPs suppressed human osteosarcoma cell metastasis by induction of EMT [72].

The development of a drug resistant phenotype can be observed after long-term treatment of osteosarcoma cells with N-BPs [73,74]. This phenotype was specific to N-BPs, and N-BP-resistant cells remained sensitive to non-N-BPs (e.g. clodronate or pamidronate) as well as being associated with a differential expression of farnesyl diphosphate synthase (FPPS), the main target of N-BPs [73]. In addition to this metabolic resistance, N-BPs increased stem cell markers such as NANOG, cMYC, OCT 4, and SOX-2 directly related to the drug resistance [74,75]. Zoledronate-induced cell resistance can by bypassed by inhibiting HSP-27 expression [76]. BPs have been also assessed in combination with other drugs or therapeutic approaches (Table 1) and showed beneficial effects. Despite the *in vitro* effect of BPs on osteosarcoma cells, no direct effects of BPs on bone sarcoma cells have been demonstrated *in vivo*.

2. Use of BPs in Ewing sarcoma

Ewing sarcoma is the second most represented bone sarcoma in terms of frequency. It affects children and adolescents, with a peak of incidence at the age of 15 years. Like osteosarcoma, it also has a marked propensity for inducing lung metastases, which are responsible for dramatically low overall survival of around 20% at 5 years [1]. Histologically, Ewing sarcoma cells are CD99 positive undifferentiated round cells. They are characterized at the molecular level by the presence of EWS/FLi gene fusion**.** Like osteosarcoma, the development of Ewing sarcoma in bone sites is associated with the recruitment of osteoclastic precursors and the activation of osteoclasts, resulting in local bone destruction. In this context, BPs have been assessed in pre-clinical *in vivo* models and revealed a significant therapeutic benefit [89-91]. N-BPs inhibited the development of primary tumors only in bone sites and not in soft tissue. They also reduced dissemination via inhibition of matrix metalloproteinases [89,90]. Zhou *et al.* compared the effect of single treatments (zoledronate, paclitaxel) with combined therapy and showed that the most effective treatment was zoledronate associated with paclitaxel [89]. Similarly, minodronate induced synergistic inhibitory activity on tumor growth when combined with doxorubicin. In combination with ifosfamide, zoledronate showed synergistic effects on *in vivo* tumor growth [90]. The aim of Ewing 2008 (Clinical Trail.gov Identifier: NCT00987636), a randomized, phase 3 clinical trial, was to study the therapeutic benefit of associating N-BPs and conventional chemotherapy. More than 900 patients with localized and disseminated Ewing sarcoma were enrolled. The final analyses are

in progress and the results will be announced soon. *In vitro,* N-BPs exhibited marked activities on cancer cells and inhibited cell proliferation with a cell cycle arrest in the S, G2/M phase [90,92,93] and additive effects when combined with conventional chemotherapeutic drugs (e.g. doxorubicin, etoposide and vincristine) [94]. N-BPs may also alter the immune response in Ewing sarcoma. Mueller *et al.* analyzed the *in vitro* antitumor activities of NK cells, and revealed that zoledronate impeded *in vitro* NK cell expansion and cytolytic function against Ewing sarcoma cells. Consequently the combination of N-BPs with adoptive transfer does not appear recommended.

3. BPs in the treatment of chondrosarcoma

With a peak of incidence at the age of 45 years, chondrosarcoma is a cartilaginous tumor characterized by malignant chondrocytes trapped in a poorly-vascularized hyaline-like extracellular matrix [95]. Depending on the histological subtype, chondrosarcomas are associated with somatic mutations in *IDH-1 or IDH-2, EXT, p53, Rb,* or *COL2A1*. Chondrosarcomas are considered to be radio- and chemoresistant tumors, and consequently the surgical approach is the first therapeutic line for low-grade entities combined with chemotherapy or radiotherapy for the highest grade tumors. Chondrosarcoma is associated with high local recurrence and a metastatic potential for high-grade tumors. Like osteosarcoma and Ewing sarcoma, osteoclasts play a key role in the pathogenesis of chondrosarcoma and the inhibition of bone resorption impaired tumor growth [96,97], and slowed down recurrent progression after intralesional curettage [96]. *In vitro* investigations demonstrated that N-BPs induced chondrosarcoma cell death in a caspase-independent manner [96,98,99] and inhibited cell invasion through decreased secretion of MMP2 [100]. Treatment of chondrosarcoma cells by N-BPs induced the production of phosphoantigen which may stimulate the expansion of Vγ9Vδ2 T cells as well as increase their cytotoxic activity [101]. A phase 1b clinical trial (Clinical Trail.gov Identifier: NCT03173976) is currently in progress to assess the safety and efficacy of neoadjuvant zoledronate in patients with resectable chondrosarcoma of any grade. Fifteen patients will be enrolled. Its completion date is estimated as July 2022.

4. Perspectives for BPs in bone sarcoma

In addition to the three main entities of pediatric and adult sarcomas described above, BPs have been assessed in other malignant and benign primary bone tumors and tumor-like lesions (Table 3). A patient suffering from an advanced sacrum chordoma with zoledronate showed a

significant pain reduction which was refractory to analgesic, opioids and antiepileptic compounds. The tumor appeared unchanged during the treatment [102]. No large clinical trial has been set up yet. Because giant cell tumors of bone are characterized by the presence of numerous and large osteoclasts, BP-based therapeutic approaches were assessed in patients [103,104]. In both studies, no serious general adverse effects were observed but adjuvant treatment with zoledronate did not prevent the local recurrence of the tumors. Aneurysmal bone cysts are aggressive and expansile bone lesions associated with hyper active osteolytic process. Pamidronate and zoledronate showed therapeutic benefits with a reduction of the bone oedema and partial or complete ossification of their lesions [105,106]. Fibrous dysplasia is a benign fibro-osseous bone disease in which the bone is progressively replaced by a fibrous tissue and increased bone resorption associated with bone pain and fragility. In this context, BPs have been also assessed in fibrous dysplasia [107-113]. BP treatments did not arrest the expanding feature of these bone lesions but showed a significant improvement in bone pain, prevented fractures. Malignant transformations have been described for all of these bone diseases. Indeed, in addition to show morphological similarities between low-grade central osteosarcoma and fibrous dysplasia [114], osteosarcoma can arise in this bone disease [115]. Similarly, osteosarcomas arising in aneurysmal bone cyst [116] or in Paget disease [117,118] have been described. Such osteosarcomas could identify a subgroup of osteosarcoma for which BPs may represent an interesting option. In a similar manner, telangietactic osteosarcoma may represent a specific subset of osteosarcoma associating giant cells enriched microenvironment [119]. BP therapies resulted in a decrease of osteoclast number and the development of a fibroblastic stroma [119]. It could be interesting to revisit the therapeutic approach of these rare osteosarcomas in the light of such observations.

As described above, from the *in vitro* and *in vivo* results of pre-clinical investigations, BPs have shown promising clinical advantages in the treatment of bone sarcomas. Unfortunately, the clinical trials have been disappointing in osteosarcoma with no improvement in clinical outcome. Thanks to their strong attraction for the bone mineral matrix, BPs have been used extensively in bone imaging activities [120]. This property has now been revisited in order to obtain functionalized BPs capable of delivering drugs to bone sites (Table 2). Various conjugation strategies are now available for functionalizing imaging agents, anti-tumor agents and nanocarriers with BPs. The readers will find more details in the excellent manuscript proposed by Xing *et al.* in the special issue on Bisphosphonates [132]. Their clinical advantages of functionalized BPs are not only to deliver high concentrations of drugs to the

tumor development site, but also to reduce the systemic effects of anti-cancer compounds, and consequently their toxicity. Some have already obtained orphan drug status and should be assessed soon in clinical trials.

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Figure Legends

Figure 1: The vicious cycle established between bone sarcoma cells and bone cells. Cancer cells secrete pro-resorptive factors such as M-CSF and RANKL. These factors recruit osteoclast precursors and activate osteoclast differentiation and activation. In turn, activated osteoclasts initiate bone resorption and release the cytokines/growth factors trapped in the mineralized extracellular matrix (e.g. TGF-β) and which stimulate cancer cell proliferation, modify their differentiation and facilitate their migration to form distant metastatic foci. Osteoblasts and mesenchymal stem cells also play a part in tumor development by direct (e.g. GAP junctions) and indirect communications (e.g. extracellular vesicles or cytokine secretion). Immune cells (e.g. macrophages) play a part in establishing a permissive local microenvironment.

Table 1. Drugs/therapies combined with bisphosphonates in osteosarcoma

BPs	Drug/therapy combined with BPS	Effects	References
Zoledronate	Paclitaxel, Doxorubicin Gemcitabine	Increased sensitity to paclitaxel, doxorubicine and gemcitabine	[77]
Zoledronate	Cisplatin	Sensitization to cisplatin	[78]
Zoledronate	TRAIL	Enhanced sensitivity to TRAIL	[79]
Zoledronate	PF4942847 (HSP90 inhibitor)	Synergistic inhibitory effect on tumor cell proliferation, metastasis development	[80]
Zoledronate	OGX-11 (clusterin inhibitor)	Synergistic activity	[81]
Zoledronate	Ursolic acid	Increased cell apoptosis	[82]
Zoledronate	RAD001 (Everolimus)	Potentiated mTOR inhibition and abolishes the resistance of osteosarcoma cells to RAD001	[83]
Minodronate	p38 inhibitor Doxorubicin	Increased efficacy of p38 inhibitor and Doxorubicin	[84]
Zoledronate Pamidronate	Radiation	Radiosensitization	[85-87]
Zoledronate	Photochemotherapy	Enhanced effect	[88]